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Exhibit C

# Intracellular degradation of prohormone-chloramphenicol-acetyl-transferase chimeras in a pre-lysosomal compartment

Ann DANOFF[1], Xiao Ping MAI[2] and Dennis SHIELDS[2]

[1] Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine, Bronx, USA
[2] Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, USA

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Small peptide hormones (less than 50 amino acids) are synthesized as larger inactive precursors. Work from several laboratories, including our own, has implicated the propeptide of various precursors in mediating intracellular transport and targeting to secretory granules. We previously demonstrated that the proregion of prosomatostatin, one of the simplest peptide hormone precursors, when fused to α-globin, enabled the globin polypeptide to be transported to the regulated secretory pathway. To identify sorting motifs in this propeptide, we have now constructed a chimera comprising the somatostatin signal peptide and proregion fused to chloramphenicol acetyl transferase (CAT) and a control protein consisting of the signal peptide fused to CAT, both of which were expressed in rat anterior-pituitary $GH_3$ cells. Both molecules were translocated into the endoplasmic reticulum (ER) efficiently and core-glycosylated on the single cryptic N-linked glycosylation site present in CAT. Surprisingly, the glycosylated propeptide-CAT and signal without CAT were degraded intracellularly with half-lives of 30 min and 90 min, respectively. Based on the kinetics of degradation, temperature sensitivity, and resistance to lysosomotrophic agents, we suggest that degradation occurred in the ER. Our data imply that the pro-region is not an *a priori* universal sorter, but only directs heterologous peptides to the secretory pathway when the passenger peptide assumes a secretion-competent conformation.

Most small peptide hormones and neuropeptides are synthesized as part of larger inactive precursors in which the hormone is flanked by pairs of basic amino acids [1]. To generate a bioactive molecule, the precursors may undergo one or several post-translational modifications, including glycosylation, proteolysis, phosphorylation, amidation and acetylation. These processing events occur in different organelles during intracellular transport, and therefore peptide-hormone precursors are useful models to study sorting through the secretory pathway [2]. Peptide-hormone-producing cells characteristically concentrate and store their secretory product in electron-dense secretory granules [3]. Upon stimulation by an extracellular signal, these granules fuse, through a calcium-dependent process, with the plasma membrane, releasing their contents into the external milieu. This type of regulated or stimulated secretion is distinct from the constitutive or basal secretion which hormone-secreting cells also perform, whereby non-hormone secretory proteins and plasma-membrane proteins are neither concentrated nor stored, but are transported via vesicles which continuously fuse with the plasma membrane [3]. Since hormone-secreting cells undergo both regulated and constitutive secretion, a mechanism must exist by which molecules are directed to the appropriate pathway.

The molecular signals that target a polypeptide hormone to the regulated secretory pathway are poorly understood, although morphological evidence has implicated selective aggregation, initiated in the *trans* Golgi network (TGN), in the sorting process [4, 5]. Current evidence suggests that in the absence of a specific topogenic signal, e.g., for retention in the endoplasmic reticulum (ER), or Golgi apparatus, or for sorting to lysosomes, secretion through the constitutive pathway occurs by default [3]. Expression of heterologous precursors in different endocrine cell lines usually results in proteolytic cleavage to the mature hormone and, in some cases, targeting to the regulated secretory pathway; e.g., preproinsulin [6], pretrypsinogen [7], preproenkephalin [8], preprorenin [9], proneuropetide Y [10], and preprosomatostatin [11, 12]. In contrast to these examples, expression of prepro-dermorphin, the precursor to the frog peptide dermorphin [13] or anglerfish preprosomatostatin-II [14] in endocrine cells lead to intracellular degradation. Several lines of evidence suggest that the pro-region contains information to target native [15–17] and some heterologous peptides to the regulated secretory pathway. For example, the pro-region of proenkephalin can target dermorphin (which is otherwise degraded intracellularly in AtT-20 mouse pituitary tumor cells) to the regulated secretory pathway where it is processed with approximately 40% efficiency [13]. 78 amino acids of the rat somatostatin propeptide were able to target the C-terminal 48 amino acids of anglerfish prosomatostatin-II (proSRIF-II) to the regulated secretory pathway [18], and the pro-region of

*Correspondence to* D. Shields, Department of Developmental & Molecular Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
*Fax:* +1 718 823 5877.

*Abbreviations.* CAT, chloramphenicol acetyl transferase; ER, endoplasmic reticulum; PCR, polymerase chain reaction; TGN, *trans* Golgi network.

*Note.* Xiao Ping Mai, deceased May 16, 1990.

von Willebrand factor precursor has been shown to mediate polymerization of its end products [19]. Since a diverse group of proteins which lack amino acid sequence similarity can be sorted to the regulated pathway, it is likely that a common structural feature, rather than a sequence is involved in targeting. However, the identity of putative sorting signals remains to be determined.

We are studying the sorting and processing of the peptide-hormone precursor prosomatostatin to identify structural domains within precursors which might function in targeting to the regulated secretory pathway [2]. In mammals, a single gene encodes a common precursor to both somatostatin 14 and somatostatin 28, the latter being a 14-residue N-terminally extended form of the tetradecapeptide [20]. However, in several species of fish, separate genes encode two distinct precursors, preprosomatostatin I and II, from which are released 14-residue and a 28-residue mature hormones, respectively [21]. To identify putative intracellular sorting domains in hormone precursors, and investigate the molecular basis for differential processing of the precursors, we expressed their cDNAs in rat anterior-pituitary growth-hormone-producing cells ($GH_3$). Previously, we demonstrated that prosomatostatin I was accurately processed in $GH_3$ cells to somatostatin 14, and efficiently targeted to the regulated secretory pathway [11]. In contrast, prosomatostatin II (which shares approximately 45% similarity with prosomatostatin I) was neither proteolytically processed nor secreted in $GH_3$ cells. Instead, it was quantitatively degraded intracellularly in a chloroquine-sensitive post-*trans* Golgi network compartment [14]. To test whether the propeptide might function as a mediator of intracellular transport, we expressed two fusion proteins in $GH_3$ cells, namely PRO-GLO, consisting of the signal peptide and proregion of somatostatin I fused to chimpanzee $\alpha$-globin (a cytoplasmic protein not likely to possess intrinsic sorting information), and SIG-GLO, in which $\alpha$-globin was fused directly to a signal peptide. Whereas the signal peptide-globin fusion protein was rapidly degraded intracellularly, with a 4–5 min half-life, the prosomatostatin I propeptide was able to rescue $\alpha$-globin from degradation, transporting approximately 35% of the chimera to the distal elements of the Golgi apparatus. Furthermore, the chimera underwent proteolytic cleavage to yield mature $\alpha$-globin which was sorted to the regulated pathway [22]. To investigate the universality of the pro-region of somatostatin I as a sorting effector, we have now constructed chloramphenicol acetyl transferase (CAT) chimeras analogous to PRO-GLO and SIG-GLO, and expressed them in $GH_3$ cells. Like SIG-GLO, SIG-CAT was degraded in the ER, although with significantly slower kinetics. Surprisingly, in contrast to PRO-GLO, the somatostatin I propeptide was unable to rescue CAT from intracellular degradation or target CAT to the regulated secretory pathway. Instead, PRO-CAT was degraded with even faster kinetics than observed for SIG-CAT turnover. These data suggest that the pro-region is not an *a priori* universal sorter, but only directs heterologous peptides to the secretory pathway when the passenger peptide assumes a secretion-competent conformation.

## MATERIALS AND METHODS

### Materials

Psi-2 cells and the plasmid pLJ were a gift from Dr R. Mulligan, The Whitehead Institute, Boston, MA. Rabbit anti-CAT serum was a gift from Dr D. Wong, this Institution. [$^{35}$S]Methionine and D-[2-$^{3}$H]mannose were purchased from Amersham Corp. Tunicamycin was purchased from Sigma, and endoglycosidase H from Boehringer Mannheim. *Taq* 1 DNA polymerase was purchased from Perkin Elmer Cetus, and Lipofectin reagent from BRL; both were used as recommended by the manufacturers.

### Methods

#### *Plasmid constructions*

SIG-CAT chimera was generated using a polymerase chain reaction (PCR) thermal cycler (Perkin Elmer Cetus) for 30 cycles (denaturation, 94°C, 2 min; annealing, 48°C, 1 min; extension 72°C, 3 min). First, two PCR fragments were generated, using primers as follows: (a) 5′-CCCGGGATCCGCAGACGCCGCCAGA-3′ and (b) 5′-TCCAGTGATTTTTTTCTCCATGCAGCTGATGGAGGC-3′ corresponding to the signal sequence of preprosomatostatin (ab), and (c) 5′-GCCTCCATCAGCTGCATGGAGAAAAAAATCACTGGA-3′ and (d) 5′-GTCGACGGATCCTTACGCCCCGCCCTGCCACTC-3′, corresponding to the coding region of CAT (cd). Following elution from an agarose gel and purification on an Elutip column, fragments ab and cd were combined and used as templates in a second PCR reaction with primers a and d, thus generating a fusion between the signal sequence of preprosomatostatin I and the coding region of CAT. The SIG-CAT chimera was subcloned into the *Bam*H1 site of the vector pLJ [22] and the cloned DNA sequence was confirmed by sequencing. PRO-CAT was constructed by conventional cloning methods using convenient restriction sites, resulting in the insertion of four additional amino acids, AlaAspProLys, at the prosomatostatin I/CAT junction.

#### *Cell culture*

Cells were grown at 37°C in an atmosphere of 7.5% $CO_2$. $GH_3$ cells were grown in Ham's F10 medium supplemented with 15% equine serum, 2.5% fetal bovine serum 2 mM glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin; Psi-2 cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum 2 mM glutamine, 25 U/ml penicillin, and 25 U/ml streptomycin [11].

#### *Infection of target $GH_3$ cells*

Infectious virus particles containing preproCAT RNA transcripts were generated by transfection of Psi-2 cells with plasmid DNA, [11]. Medium from semi-confluent G418-resistant Psi-2 cells (containing recombinant virions) was incubated with $10^6$ $GH_3$ cells for 4 h after which the medium was changed to complete Ham's F10 containing 1 mg/ml G418 as previously described [11]. $GH_3$ cells were transfected with plasmid pLJ. SIG-CAT using the lipofectin reagent, as recommended by the manufacturer. Single G418-resistant cells of both PRO-CAT and SIG-CAT were subcultured by limiting dilution in a 96-well plate, and 20 clonal lines of each were grown to mass culture.

#### *Biosynthetic labeling of cells*

35-mm dishes were seeded with $10^6$ cells, 24–72 h later the cells were washed with NaCl/$P_i$ (125 mM sodium chlo-

ride, 25 mM sodium phosphate, pH 7.6) and pulse labeled with 0.5 ml labeling medium supplemented with 2 mM glutamine, 500 μCi/ml [$^{35}$S]methionine. During the chase period, 5 μl anti-CAT sera were added to the medium. For incubation of cells at 15 °C, the chase medium was supplemented with 25 mM Hepes, pH 7.4. After the labeling and chase periods, the medium was removed, centrifuged, and the immunoprecipitable material prepared for analysis. Cells were harvested and lysed by vortexing in lysis buffer [11]. Nuclei were removed by centrifugation and the postnuclear supernatants were prepared for immunoprecipitation. Cells radiolabeled with [$^3$H]mannose were incubated for 3 h in F-10 media with the glucose reduced to 0.1 mg/ml; two 35-mm plates were each incubated with 0.5 mCi [$^3$H]mannose in RPMI containing 0.1 mg/ml glucose and the lysates pooled for further analysis. Where present, tunicamycin was used at a concentration of 5 μg/ml during prior treatment, pulse and chase periods.

*Immunoprecipitation*

To determine the intracellular levels of somatostatin-related peptides, samples were adjusted to 1% SDS and incubated at room temperature for 10 min. 10 vol. of buffer A [11] were added followed by addition of 5 μl anti-CAT sera. To assay for secreted polypeptides, the medium was adjusted to buffer A conditions by addition of 1/3 vol. of 4× buffer A. Samples were incubated with constant mixing at 4 °C for 12–24 h followed by incubation with protein-A–Sepharose at 4 °C for 3 h. Immune complexes were isolated by centrifugation, washed and analyzed by SDS/PAGE.

In vitro *transcription and translation*

The cDNAs encoding SIG-CAT, PRO-CAT and native CAT were subcloned into the transcription translation vector (pTZ 19R) downstream from the T7 RNA polymerase promoter. Following *in vitro* transcription using T7 RNA polymerase, the mRNAs were translated in the wheat-germ cell-free system in the absence and presence of microsomal membranes exactly as previously described [23–25].

## RESULTS

Previous work from our laboratory employing chimeras of preproinsulin fused to CAT [24] demonstrated that CAT can be translocated across the ER membrane efficiently *in vitro* and was efficiently glycosylated at a single cryptic *N*-linked glycosylation site. We reasoned that acquisition and modification of carbohydrate moities on CAT would provide a useful model to monitor transport through the Golgi apparatus, and thereby enable us to further investigate the role of the propeptide in mediating intracellular sorting and processing. Therefore, we constructed two fusion proteins comprising either the signal peptide alone, or the signal peptide and proregion of preprosomatostatin I fused to CAT, designated SIG-CAT and PRO-CAT, respectively (Fig. 1). A signal peptide alone has previously been shown to facilitate translocation of several normally cytoplasmic proteins, including CAT [24] and globin [26], into the ER lumen *in vitro* and *in vivo* and initiate transport through the secretory pathway [22].

Our initial experiments were aimed at characterizing the primary translation products of SIG-CAT and PRO-CAT. To this end, the *in vitro* transcription/translation products of

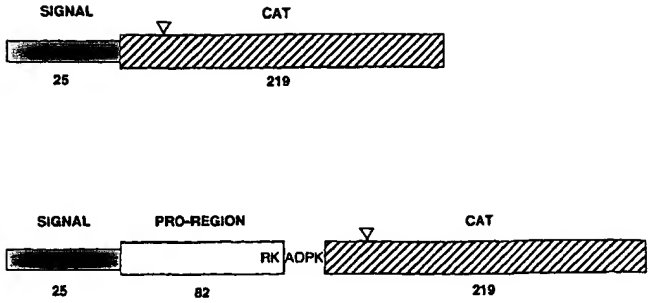


**Fig. 1. Construction of SIG-CAT and PRO-CAT chimeras.** SIG-CAT consists of the anglerfish preprosomatostatin I 25-amino-acid signal peptide (dark-box), fused to the 219-amino-acid, coding sequence of CAT (hatched box). PRO-CAT contains the signal peptide and 82-amino-acid proregion of preprosomatostatin I (clear box), fused to the coding sequence of CAT; four additional residues (AlaAspProLys; ADPK) resulting from subcloning were inserted at the N-terminus of CAT. These additional amino acids have previously been shown to have no effect on endoproteolytic processing [22]. The single set of paired basic amino acids ArgLys in prosomatostatin are the site of endoproteolytic cleavage of the native precursor (RK). The arrowhead indicates the single site of potential N-linked glycosylation (Asn34GluThr) in CAT.

these chimeric cDNAs were compared to those synthesized in rat anterior-pituitary $GH_3$ cells (Fig. 2). Translation of SIG-CAT mRNA resulted in a major product ($M_r$ 24000) of the predicted size for a signal peptide-CAT fusion protein. A minor translation product ($M_r$ 22000) which migrated on SDS/PAGE with native CAT (219 amino acids) probably corresponded to internal initiation at the first methionine residue following the signal peptide of SIG-CAT. When SIG-CAT mRNA was translated in the presence of microsomal membranes three polypeptides were evident. The minor band ($M_r$ 25000) corresponded to core glycosylated CAT lacking its signal peptide, and had the identical mobility to glycosylated SIG-CAT expressed *in vivo*. When $GH_3$ cells expressing SIG-CAT were incubated in the presence of tunicamycin, an inhibitor of N-linked glycosylation, the 25-kDa polypeptide was absent, demonstrating that it was a glycosylated form of CAT. This was confirmed by its suceptibility to digestion with endoglycosidase H. The fastest migrating polypeptide corresponded to SIG-CAT lacking its signal peptide, and had the same mobility as SIG-CAT synthesized *in vivo* in the presence of tunicamycin. The third translation product was residual unprocessed SIG-CAT. These data showed that the signal peptide was sufficient to effect CAT translocation into microsomal membranes *in vitro* albeit rather inefficiently. Most significantly, SIG-CAT was very efficiently translocated into the ER *in vivo* as determined by cleavage of its signal peptide and aquisition of N-linked carbohydrate.

Comparison of the *in vitro* translation products of PRO-CAT (Fig. 2B) with those expressed in $GH_3$ cells showed that it was also efficiently translocated into the ER and core glycosylated *in vivo*. Translation of prePRO-CAT mRNA resulted in a major product of the expected size, $M_r$ 39500. In addition, a $M_r$ 22000 polypeptide corresponding to the product of internal initiation at the first methionine in CAT was also evident; this polypeptide migrated with native CAT synthesized in $GH_3$ cells. When prePRO-CAT mRNA was translated in the presence of microsomal membranes an additional polypeptide $M_r$ 33000, corresponding to the chimera lacking its signal peptide was evident. This polypeptide had the same mobility as non-glycosylated PRO-CAT synthe-

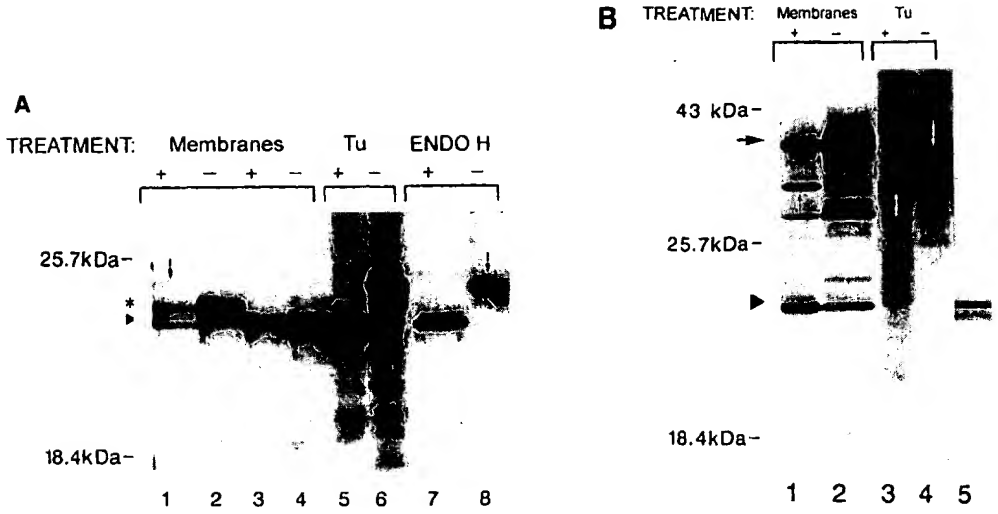


**Fig. 2. Characterization and comparison of SIG-CAT and PRO-CAT translation products synthesized *in vitro* and *in vivo*.** Plasmids pTZ19R. SIG-CAT and pTZ19R. PRO-CAT were transcribed *in vitro*, the RNA translated in the wheat-germ protein-synthesizing system containing [$^{35}$S]methionine in the presence (+) or absence (−) of microsomal membranes. $GH_3$ cells expressing SIG-CAT or PRO-CAT (Materials and methods) were pulse labeled with [$^{35}$S]methionine for 10 min in the absence or presence of tunicamycin (Tu). The cell lysates were incubated with anti-CAT sera and aliquots of the immunoprecipitated material were either prepared directly for electrophoresis (A, lanes 5, 6, 8; B, lane 4), or first treated with Endo H prior to SDS/PAGE (A, lane 7). (A) SIG-CAT. Lanes 1−4, *in-vitro*-translation products; lanes 5−6, products synthesized in $GH_3$ cells. Lane 1, SIG-CAT synthesized in the presence of microsomal membranes; (↓), core glycosylated CAT; (*), signal peptide-CAT; (▶), non-glycosylated CAT translocated into the ER, lacking its signal peptide. Lane 2, products synthesized in the absence of microsomal membranes; (*), signal peptide CAT; (▶), CAT in which internal translation initiation occurred at the first methionine residue following the signal peptide i.e. Met1 of CAT. Lane 3, products of native CAT mRNA plus microsomal membranes. Lane 4, products of CAT mRNA minus microsomal membranes. Lane 5, SIG-CAT immunoreactive polypeptides synthesized in the presence of tunicamycin. Lane 6, SIG-CAT immunoreactive products. Lane 7, CAT-immunoreactive polypeptides synthesized in $GH_3$ cells digested with Endo H prior to SDS/PAGE. Lane 8, as lane 7 except that the immunoprecipitates were treated with buffer alone prior to SDS/PAGE (the downward arrow corresponds to core-glycosylated (Endo H sensitive CAT). (B) PRO-CAT. Lanes 1 and 2, *in-vitro*-translation products synthesized in the presence and absence of microsomal membranes. Lanes 3−5, polypeptides synthesized in $GH_3$ cells. Lane 1, (⇒), unprocessed preproCAT; upward-pointing arrow, non-glycosylated proCAT translocated into the ER; (▶), internal initiation of preproCAT mRNA at the first methionine residue of CAT. Lane 2, (⇒), preproCAT; (▶), internal initiation of translation product. Lane 3, PRO-CAT synthesized in the presence of tunicamycin; upward-pointing arrow, non-glycosylated proCAT (this migrated with proCAT synthesized in the presence of microsomal membranes, lane 1). Lane 4, Core glycosylated PRO-CAT (downward-pointing arrow). Lane 5, products of native CAT mRNA.

sized *in vivo* in the presence of tunicamycin. Although PRO-CAT was core glycosylated *in vivo*, it was a poor substrate for *in vitro* glycosylation since no band corresponding to the $M_r$ 38000 glycosylated form was evident. Most importantly, these data demonstrate that PRO-CAT, like SIG-CAT was efficiently translocated into the ER lumen in $GH_3$ cells.

Our next experiments were aimed at determining if the preprosomatostatin I signal peptide was sufficient to facilitate CAT transport through the secretory pathway. Cells were pulse labeled for 10 min with [$^{35}$S]methionine chased for up to 4 h at 37°, and the intracellular and secreted CAT-immunoreactive polypeptiies analysed by SDS/PAGE (Fig. 3). A major CAT-immunoreactive polypeptide of approximate $M_r$ 25000 corresponding to SIG-CAT lacking its signal peptide, and containing core N-linked carbohydrate (Figs 2 and 5), was evident intracellularly up to 180 min of chase, thereafter the intensity of this material diminished. In addition a second CAT-immunoreactive polypeptide ($M_r$ 22000) was also evident, which corresponded to SIG-CAT lacking both its signal peptide and core carbohydrate (Fig. 2). Consistent with this assignment, the $M_r$ 25000 polypeptide was the only CAT-immunoreactive molecule radiolabeled in the presence of [$^3$H]mannose. It is noteworthy that the non-glycosylated form of CAT disappeared much more rapidly than the glycosylated polypeptide. Most significantly, no CAT polypeptides were detected in the culture medium even following 4 h of chase. Based on our previous observations on the turnover of SIG-GLO and prosomatostatin II in $GH_3$ cells [14, 22] we suspected that the SIG-CAT molecule might be degraded in the ER. Consistent with this idea, both forms of CAT were evident following 3 h of chase at 15°C, a temperature that prevents protein transport out of the ER.

The preceding data suggested that a signal peptide alone was insufficient to allow CAT to be transported through the complete secretory pathway, although we cannot exclude the possibility that partial transport occurred. Based on previous data from our laboratory and others [2], we hypothesized that the somatostatin propeptide would mediate intracellular CAT transport and targeting to the regulated secretory pathway. Following 10 min pulse-labeling with [$^{35}$S]methionine a 38-kDa CAT-immunoreactive polypeptide was present intracellularly (Fig. 3B); this corresponded to core glycosylated PRO-CAT, from which the signal peptide had been cleaved (Fig. 2B) Surprisingly, immunoreactive PRO-CAT also disappeared during the chase period (Fig. 3B) and no CAT immunoreactive material was detected in the medium (data not shown). Furthermore, the kinetics of PRO-CAT disappearance were significantly faster than those of SIG-CAT, half-lives of approximately 30 min and 90 min, respectively (Fig. 4). Similar to SIG-CAT, there was no degradation of

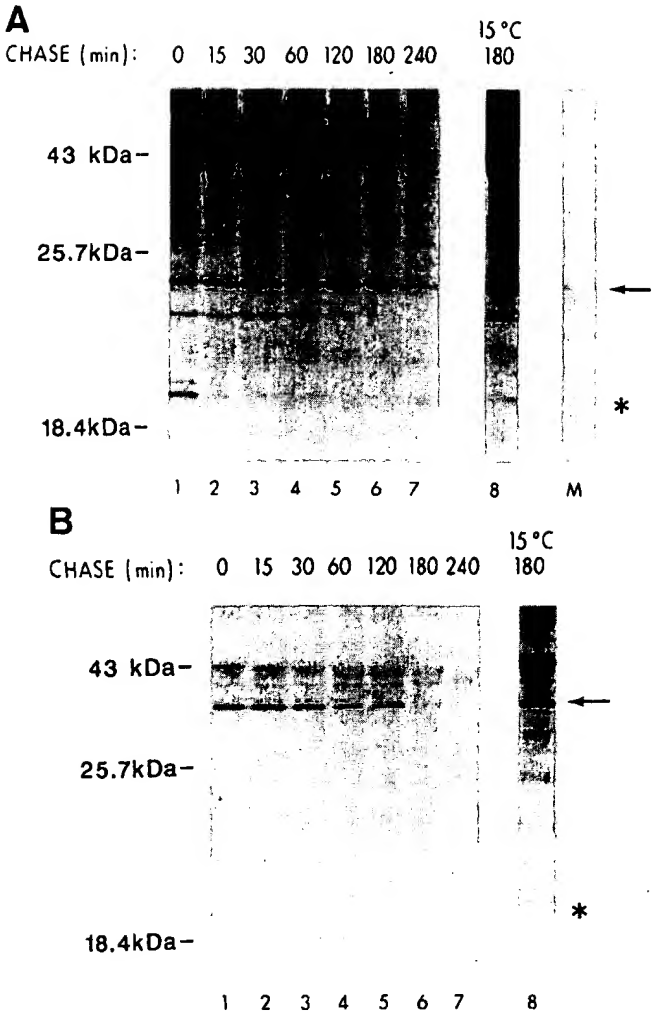


**Fig. 3. *In vivo* degradation of SIG-CAT and PRO-CAT.** (A) $GH_3$ cells expressing SIG-CAT were pulse labeled with [$^{35}$S]methionine for 10 min and chased for the indicated times at 37°C (lanes 1–7) in the presence of 5 mM methionine. At each time point the cells were lysed, treated with anti-CAT sera and the immunoprecipitable material analyzed by SDS/PAGE followed by fluorography. Lane 8, CAT-immunoreactive polypeptides following 3-h chase at 15°C. Lane M, CAT-immunoreactive material radiolabeled with [$^3$H] mannose (⇐). (B) PRO-CAT. $GH_3$ cells expressing PRO-CAT were pulse-labeled and chased at 37°C (lanes 1–7) exactly as described for SIG-CAT cells (above). Lane 8, PRO-CAT-immunoreactive polypeptides synthesized following 3-h chase at 15°C. (*) An unidentified polypeptide that may be a CAT degradation product.

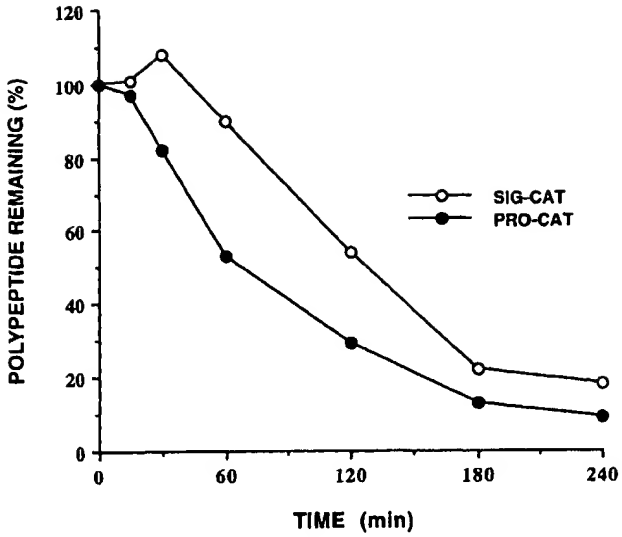


**Fig. 4. Kinetics of SIG-CAT and PRO-CAT degradation *in vivo*.** The polypeptides corresponding to SIG-CAT and PRO-CAT in the fluorograms of Fig. 3 were analyzed by densitometry (Materials and Methods). Percentage remaining corresponds to the fraction of material present at each time point compared to that present at the end of the 10-min pulse (100%). Tu, tunicamycin.

PRO-CAT upon incubation at 15°C suggesting that degradation required exit from the ER or that the proteases involved were temperature sensitive.

If SIG-CAT and PRO-CAT were degraded in the proximal elements of the secretory pathway i.e. ER, intermediate compartment, or *cis* Golgi apparatus, then both these molecules should remain sensitive to digestion by endoglyosidase H. Conversely, if SIG-CAT and/or PRO-CAT reached the late Golgi, then they would be expected to have acquired Endo H resistance. Upon incubation with Endo H the $M_r$ 25000 form of SIG-CAT was reduced in size and migrated on SDS/PAGE with SIG-CAT synthesized in the presence of tunicamycin (Fig. 5A). Even after 180 min of chase all the residual SIG-CAT remained Endo H sensitive, suggesting it had not been transported beyond the *cis* Golgi (Fig. 5B) Similarly, at each time point up to 120 min of chase, PRO-CAT was also Endo H sensitive and the $M_r$ 38000 form migrated faster with an apparent $M_r$ of approximately 33000 (Fig. 5B). Thus, both SIG-CAT and PRO-CAT remained Endo H sensitive even following a long (>2 h) chase period, suggesting that neither molecule had been efficiently transported beyond the *cis* Golgi apparatus and that degradation occurred early in the secretory pathway.

To test the idea that SIG-CAT and PRO-CAT were degraded prior to reaching lysosomes, cells were incubated in the presence of high concentrations of the weak base chloroquine (Fig. 6) which we previously showed inhibited prosomatostatin II degradation in an acidic intracellular compartment [14], or leupeptin, an inhibitor of lysosomal serine and thiol proteases such as cathepsin B. Neither of these reagents inhibited the intracellular degradation of SIG-CAT and in contrast to the effect of chloroquine on prosomatostatin II degradation, there was no secretion of CAT in response to either treatment. Similarly, neither chloroquine nor leupeptin had a significant effect on the degradation of PRO-CAT (Fig. 6B). Taken together these data suggest that both SIG-CAT and PRO-CAT are degraded in a prelysosomal compartment, either the ER or early Golgi apparatus.

## DISCUSSION

Evidence from a number of laboratories, including our own, (reviewed in [2]) has suggested that the propeptides in peptide hormone precursors may contain targeting information for sorting to the regulated secretory pathway. Expression of several chimeric proteins in neuroendocrine cells resulted in the propeptide conferring a dominant phenotype with respect to sorting and processing. For example prosomatostatin-GLO was sorted to the regulated pathway in rat $GH_3$ cells and approximately 40% of the chimera was processed to α-globin [22], whereas, in the absence of the pro-

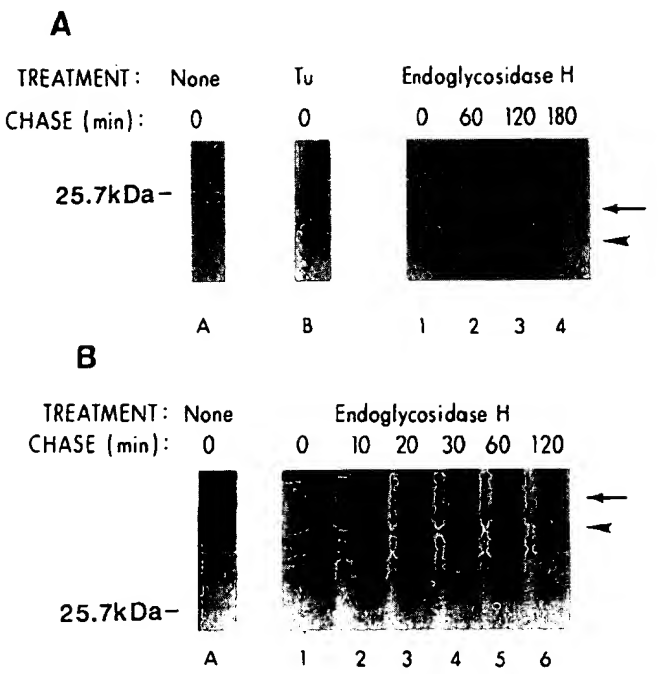


**Fig. 5. SIG-CAT and PRO-CAT are sensitive to endoglycosidase H prior to degradation.** (A) SIG-CAT. $GH_3$ cells expressing SIG-CAT were pulse labeled with [$^{35}$S]methionine for 10 min, chased for the indicated times and the cell lysates treated with anti-CAT sera. LaneA, glycosylated CAT present after 10 min of pulse label, (0 min chase). Lane B, non-glycosylated CAT present after 10 min pulse label in the presence of tunicamycin. Lanes 1–4, at the indicated times, the CAT-immunoreactive polypeptides were treated with Endoglycosidase H (Materials and Methods) prior to preparation for SDS/PAGE. (⇐), Migration of glycosylated CAT; (◀), mobility of non-glycosylated CAT. (B) PRO-CAT. $GH_3$ cells expressing PRO-CAT were pulse labeled with [$^{35}$S]methionine and chased for the indicated times. At each time point cell lysates were treated with anti-CAT sera followed by Endoglycosidase H digestion (lanes 1–6). Lane A, core glycosylated PRO-CAT present after 10 min of pulse labeling (0 min chase). (⇐), Migration of glycosylated PRO-CAT; (◀), migration of de-glycosylated PRO-CAT.

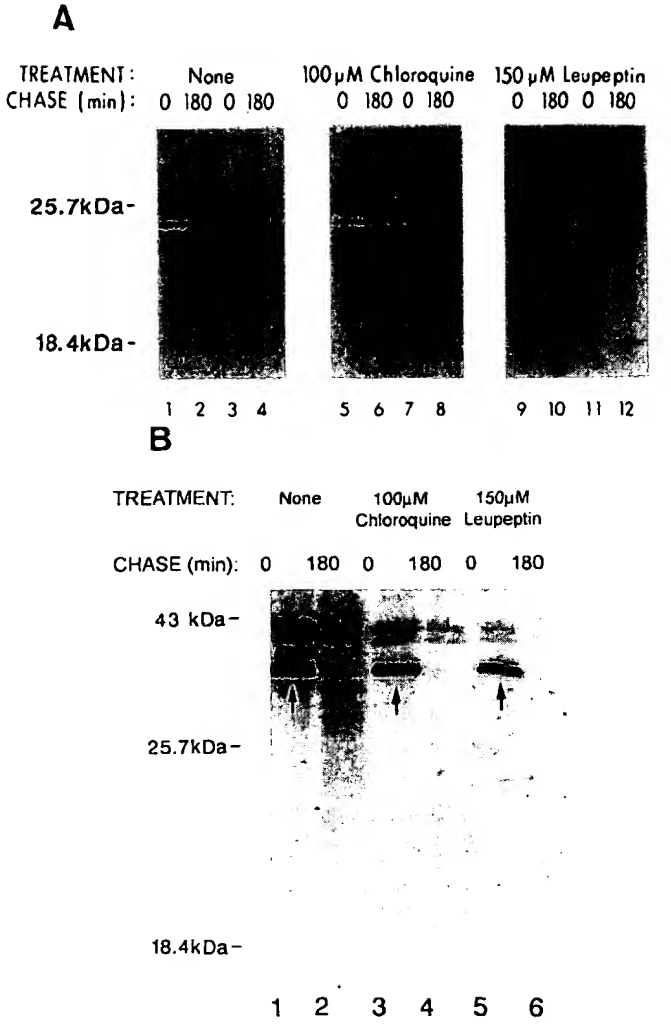


**Fig. 6. Degradation of SIG-CAT and PRO-CAT occurs in a pre-lysosomal compartment.** (A) SIG-CAT. $GH_3$ cells were pulse labeled for 10 min and chased for 0 min or 180 min (lanes 1–4), in the presence of 100 μM chloroquine (lanes 5–8) or 150 μM leupeptin (lanes 9–12). The intracellular material (lanes 1, 2, 5, 6, 9, 10) and medium (lanes 3, 4, 7, 8, 11, 12) were then analyzed for CAT-immunoreactive polypeptides. (B) PRO-CAT. $GH_3$ cells were pulse labeled for 10 min and chased for 0 min and 180 min (lanes 1, 2), in the presence of chloroquine (lanes 3, 4) or leupeptin (lanes 5, 6). At each time point the cell lysates were treated with anti-CAT sera.

peptide the α-globin was degraded, most likely in the ER or intermediate compartment. Likewise anglerfish prosomatostatin II, which was poorly and incorrectly cleaved in AtT-20 cells, could be routed to the regulated pathway and cleaved correctly when 48 amino acids of the C-terminus were fused to the first 78 amino acids of rat prosomatostatin and expressed in these cells [18]. Analogous studies demonstrated that when the precursor to frog demorphin was expressed in AtT-20 cells it was degraded [13]. However, expression of a proenkephalin-demorphin chimera in these cells showed that the enkephalin propeptide rescued demorphin from degradation resulting in sorting to the regulated pathway, with approximately 40% processing efficiency. Perhaps the most compelling evidence that a propeptide may have sorting information comes from the *in vivo* expression of a pro-α-factor-somatostatin hybrid, which was transported and processed with an efficiency and kinetics that were similar to native prepro-α-Factor [27, 28]. Most significantly, these studies also demonstrated that the propeptide rather than the mature hormone was recognized by the KEX2p and STE13p processing enzymes. Taken together these studies suggest that propeptides contain intracellular sorting information. In general the amino acid sequences of most prohormones are not conserved, and no conserved structural motifs have been identified in peptide hormone precursors; consequently, it is unlikely that a continuous sequence is responsible for sorting. The nature of this information remains elusive i.e., whether it resides in the conformation of the whole propeptide subdomain remains to be determined.

The C-terminal portion of many prosomatostatins is conserved between species, although overall the similarity between these prohormones is only about 50% [29]. However, given that there is some conservation of amino acid sequence in prosomatostatin and the observations that the somatostatin propeptide was able to transport heterologous peptides including α-globin [22], prosomatostatin II [18] and hirudin (Ory, D., and Mulligan, R. C., personal communication), through the secretory pathway, we investigated whether the propeptide was able to act as a universal sorting sequence.

Surprisingly, the pro-region of somatostatin was unable to sort the PRO-CAT chimera to either the regulated or constitutive secretory pathway. Instead, like the SIG-CAT chimera, it was degraded intracellularly most likely in the ER, intermediate compartment or early Golgi apparatus (Figs 2 and 3). Furthermore, PRO-CAT was degraded at about twice the rate of SIG-CAT. We speculate that since native CAT normally forms a tetramer, in neither of these chimeras was CAT able to assume its native conformation, and therefore residues that are normally buried within the hydrophobic core of the enzyme were now exposed on the surface of the molecule. Consequently, upon translocation into the ER lumen each chimera was recognized as misfolded, presumably by the same degradation enzyme(s) that are responsible for turnover of T-cell-receptor subunits [30–32], 3-hydroxy-3-methylglutaryl coenzyme A reductase [33, 34] and the asialoglycoprotein receptor [35, 36]. Thus these data imply that even though the somatostatin propeptide is able to confer sorting information on some molecules not normally targeted to the secretory pathway, it cannot function as a universal targeting sequence except when the passenger polypeptide is also able to fold into a stable structure compatible with ER to Golgi transport.

Most examples of proteins that are degraded in the ER have been those of individual subunits of oligomeric membrane proteins [32] or polytopic membrane proteins [33], although several examples of secretory protein degradation have also been described [37, 38]. However, both CAT and α-globin, which normally reside in the cytoplasm, were degraded upon translocation to the ER, with kinetics significantly faster than those of most membrane proteins. In this context, Su et. al. [38] recently demonstrated that yeast native pro-α-factor expressed in $GH_3$ cells remained Endo H sensitive and was also degraded in the ER/early Golgi apparatus. The authors suggest that trimming of specific mannose residues by mammalian mannosidases absent in yeast, may generate an atypical core-carbohydrate structure that confers protease sensitivity on this foreign protein. Consistent with this hypothesis, preservation of the $Man_{8-9}$ structure, by treatment of cells with deoxymannojirimycin enhanced the half-life of pro-α–factor. In the data presented here, both SIG-CAT and PRO-CAT were also core glycosylated efficiently *in vivo* (Figs. 2 and 3) and degraded following an approximately 15-min lag (Fig. 3) perhaps indicating that initial carbohydrate trimming preceded degradation, as suggested by Su et. al. [38]. Similar to our earlier data on globin degradation and that for several membrane proteins, we did not observe peptide intermediates at any time points, even though the turnover of SIG-CAT was relatively slow compared to that of SIG-GLO [22]. It might be argued that the disappearance of CAT-immunoreactivity was due to its post-translational modification such as glycosylation and consequently our anti-CAT sera were unable to recognize it. This is unlikely since our present data and previous studies [24] showed that it was able to recognize glycosylated and non-glycosylated forms of CAT. Although protein misfolding and the consequent exposure of normally buried domains has been implicated in determining protein degradation in the ER, the actual mechanism by which some proteins tumover whereas others sustain prolonged half-lives and bind to chaperone-like molecules such as BiP is unclear [37]. In preliminary experiments (Danoff, A. and Shields, D., unpublished results) neither SIG-CAT or PRO-CAT were found in association with BiP. In addition, the protease(s) involved in ER-protein degradation have not been characterized in detail, although Inoue et. al. [34] have implicated calcium-dependent cysteine proteases in this pathway, and Urade et al. [39, 40] have characterized a $M_r$ 60000 protease with sequence similarity to phosphoinositol-lipid-specific phospholipase C-α in the turnover of endogenous ER proteins. Based on the kinetics of SIG-CAT and PRO-CAT degradation and their turnover in the presence of inhibitors of lysosomal enzymes, we speculate that these polypeptides are likely substrates for this same degradation pathway. At present, it is unclear which domains in the CAT molecule are incompatible with correct folding or are recognized as potential substrates for proteolysis; the construction and expression of additional chimeras will address this point.

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